with 600N. 8mm cartilage plugs containing the 4mm impacted core and 4mm immediately adjacent non-impacted ring were removed and cultured with or without P188 (8mg/ml) alone or in combination with OP-1 (100ng/ml). For metabolic studies, tissue explants were cultured with P188 and OP-1 for 48 hours, after which the media were replaced with plain media contained ITS and the cultures were continued for up to 14 days. Cell survival with live/dead assay, apoptosis with Tunel assay and structural integrity with Safranin O staining were assessed at days 0, 2, 7, and 14. Mechanistic studies were undertaken in order to assess the mode of action of P188 via its regulation of IL-6 and MAPK mediated signaling pathways. In this part of the study, tissue explants were cultured in the presence of P188 only for 20 min, 1 hour, and 24 hours, after which the cells were lysed and total lysates were blotted with the antibodies against phosphorylated and non-phosphorylated forms of ERK, JNK, P38 kinase, Stat1, Stat3, ATF-2, and GSK3. In all experiments the data were compared to the corresponding non-treated impacted control and to normal non-impacted cartilage.
Results: A single impact to human articular cartilage resulted in cell death at the impaction site and radial progression of apoptosis to adjacent ring. P188 alone promoted cell survival by reducing cell death more than 2-fold ($p<0.05$) in the core and about 30% in the ring as compared to all untreated controls. It also inhibited expansion of apoptosis in the ring especially within first 7 days post impaction (7.5% Tunel-positive cells vs 46% in the untreated control; $p<0.01$). Effect of OP-1 on P188-mediated responses was primarily accumulated by day 14, where under the combined treatment there was a tendency towards enhanced cell survival and reduced apoptosis, and statistically significant improvement of histological appearance of cartilage in both core and ring areas ($P<0.04$ and $P<0.01$ respectively) in comparison to the corresponding areas treated with P188 alone. We also found that injury to human cartilage resulted in the activation of IL-6 signaling, stress related pathway, namely P38, and apoptosis related GSK3 activation. We identified here that P188 mediated its protective effects on cell survival and cartilage integrity through the inhibition of phosphorylation of MAPK/ERK, JNK, and p38 and attenuation of IL-6 signaling via inhibition of Stat1 and Stat3; furthermore, phosphorylation of ATF2 (downstream in P38 pathway) was also affected. In addition, P188 was able to inhibit phosphorylation of GSK3.
Conclusions: Observed results identified novel mechanisms of P188 action and suggest its therapeutic potential alone or in combination with anabolic agent OP-1 in preventing progressive cartilage degeneration and the development of post-traumatic OA.

217

CHROMATIN PROTEIN HMGB2 REGULATES ARTICULAR CARTILAGE SURFACE MAINTENANCE VIA BETA-CATENIN PATHWAYS

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Purpose: The superficial zone (SZ) of articular cartilage is critical in maintaining tissue function and homeostasis and represents the site of the earliest changes in osteoarthritis. Mechanisms that regulate the unique phenotype of SZ chondrocytes and maintain SZ integrity are unknown. We have recently demonstrated that expression of the chromatin protein HMGB2 is restricted to the SZ of articular cartilage suggesting a transcriptional network involving HMGB2 that regulates SZ cell phenotype. This study addresses potential interactions between HMGB2 and the Wnt/β-catenin pathway in regulating SZ cells.
Methods: Activation Wnt/β-catenin signaling in articular cartilage was assessed by immunostaining for galactosidase in TOPGAL reporter mice at 3, 6, 9 and 12 months of age, and this was compared with HMGB2 expression. Functional interactions of β-catenin and HMGB2 on cyclin D1 and TOPflash promoters which are responsive to Wnt/β-catenin activation was examined by luciferase assay, and molecular interactions between β-catenin, Lef-1 and HMGB2 were examined by GST-pull down assays. Gel shift assays were used to determine binding specificity and interactions of HMGB2 and Lef-1. To further test the functional significance of β-catenin signaling, we conditionally inactivated β-catenin in chondrocytes isolated from β-catenin floxed mice (Ctnnb1flox/flox) with adenovirus-GFP-Cre and performed apoptosis assays.
Results: We found that the Wnt/β-catenin pathway is active specifically in the SZ in normal mouse knee joints and colocalizes with HMGB2. Both, Wnt signaling and HMGB2 expression decrease with aging in mouse joints. Our molecular studies show that HMGB2, Lef-1 and β-catenin form a tri-molecular complex on a promoter containing a Lef-1 motif. This complex enhances the binding of Lef-1 to its target sequence and potentiates transcriptional activation of the Lef-1-β-catenin complex. Furthermore, conditional deletion of β-catenin in cultured mouse chondrocytes induced apoptosis.
Conclusions: These findings define a pathway where protein interactions of HMGB2 and Lef-1 enhance Wnt signaling and promote SZ chondrocyte survival. Loss of the HMGB2-Wnt signaling interaction is a new mechanism in aging-related cartilage pathology.

218

CANONICAL WNT EXPRESSION IN THE SYNOVIUM INDUCES OA-LIKE CARTILAGE DEGENERATION

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Purpose: In osteoarthritis (OA), cartilage damage is one of the main pathological features. There is, however, a significant involvement of the synovium in a large proportion of OA-patients. The mechanism through which the synovium contributes to OA pathology is not yet known. In cartilage, it is proposed that developmental processes important in embryonic development are reactivated. Associations have been identified of the occurrence of OA with polymorphisms of genes from the wnt/β-catenin pathway, a pathway that is involved in cartilage development. The aim of the present study is to investigate the contribution of the synovium to OA pathology via wnt signaling.
Methods: A longitudinal expression analysis was performed in 2 murine models for OA, one with clear synovial involvement, collagenase induced (CIOA), and the spontaneous OA model in STR/ort mice, which shows less synovial involvement. CIOA was generated by intra-articular injection of collagenase, which induces joint instability. Synovial expression of components of the wnt signaling pathway was determined at several time points. From these results, targets were selected for the generation of adenoviral vectors to overexpress specific genes. To study the effect of these genes on chondrocytes, human chondrocytes were isolated from cartilage that was obtained from joint replacement surgery. In vivo effects were determined by intra-articular injection of the viral vectors and determining joint pathology by histology at several time points after injection.
Results: Strong upregulation of the canonical wnts wnt16 (up to 256-fold) and wnt2b (up to 90-fold) was found in both models, although stronger in CIOA. Expression in the synovium was clearly higher compared to the cartilage. Clear intracellular accumulation of β-catenin was found in both synovium and cartilage, which

indicates the activation of wnt/β-catenin in both tissues. Wnt-1 induced signaling protein (WISP1), a protein downstream canonical wnt signaling, was highly expressed in the synovium as well, again indicating activation of this pathway. To determine whether canonical wnt expression in the synovium has the potency to cause cartilage damage, the canonical wnt wnt8a was overexpressed specifically in the synovium by intra-articular injection of an adenoviral vector. At day 1 and 3, no significant differences were observed in the cartilage from wnt8 overexpressing knee joints compared to joints transfected with control virus. Remarkably, at day 7, a strong induction of cartilage pathology was observed at the medial margin of the medial tibial plateau (Figure 1), a preferential site for the start of cartilage damage in our models. This shows that expression of canonical wnt in the synovium causes cartilage degeneration.

Due to their size, wnt proteins and WISP1 can reach the chondrocytes in the cartilage matrix and may alter the chondrocyte phenotype. Overexpression of wnt8, wnt16 and WISP1 in human chondrocytes led to the a significant increase within 14 days of Collagen type I, and a significant decrease of Collagen type II, suggesting degeneration of the chondrocyte phenotype.

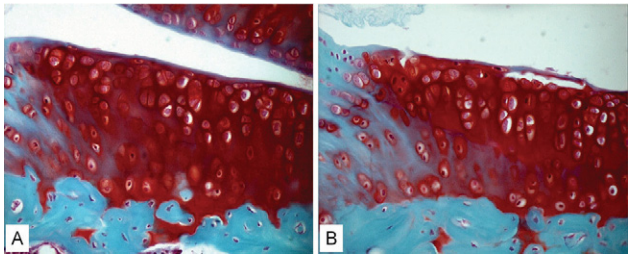

Figure 1. Focal OA-like cartilage degradation on the medial tibia after 7 days over-expression of wnt8a (B) in murine knee joints. Control virus did not induce pathology (A).

Conclusions: Canonical wnt expression and subsequent WISP1 is increased in the synovium during experimental OA. This synovial expression may lead to the degradation of cartilage, possible by dedifferentiation of the articular chondrocyte phenotype. This indicates synovial wnt expression as a potential target for OA therapy.

219

CHONDROCYTE RESPONSIVNESS TO LEPTIN IS STRONGLY DEPENDENT ON THE BODY MASS INDEX OF PATIENTS WITH OSTEOARTHRITIS

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Purpose: Recent studies that have examined the relationship between body composition and cartilage structure indicate that metabolic factors associated with adiposity may contribute to the development and the progression of osteoarthritis (OA). Among adipose-derived proteins, namely adipokines, leptin has been identified as an important factor able to modulate chondrocyte functions. Although the physiological activity of leptin is to reduce food intake, obesity is characterized by an elevated systemic level of leptin which fails to decrease body weight, suggesting a leptin resistance in obese individuals. The current study has been therefore undertaken to determine whether hyperleptinemia found in the joint from obese OA patients induces also a defect in the action of leptin in chondrocyte.

Methods: Chondrocytes isolated from OA patients with various BMI ranging from 22 to 47 kg/m^2 were treated with 100 or 500 ng/ml of leptin. The expression of cartilage-specific components (aggrecan, type 2 collagen), as well as regulating factors (IGF-1, TGFβ, MMP-13, TIMP 2), was investigated by quantitative real-time PCR to evaluate chondrocyte responsivness to leptin.

Results: Addition of leptin to human OA chondrocytes up-regulates the expression of the genes encoding cartilage-specific components and regulating factors. However, the effect of the adipokine was shown to be strongly dependent on the concentration and the BMI of the patients. When both groups of OA patients were compared, chondrocytes obtained from normal or overweight patients (BMI30 kg/m^2). Based on this BMI-dependent leptin dose-response of chondrocytes, we demonstrated that the stimulating effect of leptin at 100 ng/ml was negatively related with the BMI of the patients while a positive association between chondrocyte responsivness and BMI was found at 500 ng/ml of leptin. Beside, the growth factor induced by leptin was also dependent on BMI. IGF-1 was up-regulated in chondrocytes collected from normal or overweight patients while mRNA level of TGFβ was increased in leptin-treated chondrocytes provided by obese patients. Moreover, the gene encoding MMP-13 was identified as a target of leptin for chondrocytes originated from obese patients only.

Conclusions: The current study showed that the BMI of OA patients changed the leptin dose-response of chondrocytes and indicated that chondrocytes from obese patients required elevated levels of leptin to be responsive. In addition, the BMI-dependent effect of leptin for the expression of growth factors and MMP-13 suggests that the adipokine may contribute to the fast progression of OA in obese individuals.

220

ANALYSIS OF THE SECRETOME OF HUMAN ARTICULAR CHONDROCYTES INDUCED BY ACTIVATION OF TOLL-LIKE RECEPTOR 2

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Purpose: Toll-like receptors (TLR) are the major mediators of the innate immune response. In monocytes, macrophages and dendritic cells, the major effector cells, the primary response to TLR activation is the induction of inflammatory cytokines, chemokines and a varitety of matricellular proteins, which have been implicated in wound healing and tissue repair. In articular cartilage TLR activation, particularly that of TLR2, leads to expression of matrix metalloproteases and thus may contribute to cartilage erosion in osteoarthritis. However, the complete secretome of articular chondrocytes in response to TLR ligands has not been characterized. The purpose of this study was to analyse the proteins induced following activation of TLR2 and to determine if some of these components were present in human articular cartilage with early degenerative changes.

Methods: Human chondrocytes were exposed for 24 h to the TLR2 ligand peptidoglycan or TNF-α. Secreted proteins were analysed by mass spectrometry. The cytokine profile was analysed using a cytokine array. In addition, TNF-α levels in culture media were determined by Elisa assay. Induction of MMP3, MMP13, the Chi-lectins Chi3L1 and 2, and complement component C3 was further analysed by western blotting. Cartilage harvested from patients undergoing joint replacement (n=35, age range: 69 to 101 years old, 31 females, 4 males). following femoral neck fracture was extracted with guanidine. and e Extracts were analysed by western blotting for the presence of Chi3L1 and Chi3L2, and their levels were quantitated densitometrically and normalized to standards of purified protein included in each gel.

Results: Stimulation with the TLR2 ligand peptidoglycan led to increased production of the matrix metalloproteases MMP3 and